MULTIPLEXED CAPILLARY ELECTROPHORESIS SYSTEMS

Cross-reference to related applications

5   This application claims priority to United States Provisional Patent Application Serial No. 60/372,359, filed on April 12, 2002; and is a continuation-in-part of application Serial No. 09/946,396, filed September 5, 2001, which claims priority to United States

10   Provisional Patent Application Serial No. 60/230,507 and 60/230,508, both filed on September 6, 2000; the entire disclosures of which are incorporated herein by reference.

15   Field of the Invention

Instrumentation, and accompanying system for multiplexed separation and detection of proteins, peptides and biomolecules by electrophoresis and

20   related techniques.

Background of the Invention

Electrophoresis is one of the most widely used

25   separation techniques in the biological sciences. The use of electrophoresis can be performed in any one of several formats, including slab gel electrophoresis, paper electrophoresis, and capillary electrophoresis. While slab gel electrophoresis is the most commonly

30   used of these formats, capillary electrophoresis has been gaining in popularity since its introduction by Bushey and Jorgenson in 1981 (*Anal. Chem. 55*, 1198-1302). The reason for this is that slab gel

electrophoresis is time consuming and suffers from
gel-to-gel irreproducibility. On the other hand,
capillary electrophoresis (CE) is fast, and lends
itself more readily to automation, and is generally
5   more reproducible from lab to lab. Although
multiplexed CE separation of nucleic acid molecules is
becoming routine, this has not been the case for
proteins or other biomolecules, because these are more
difficult separations, as there are a greater variety
10   of chemical challenges.

In addition to the existance of several formats
of electrophoresis, there exist also a variety of
modes, including free zone electrophoresis, gel
15   electrophoresis, and isoelectric focusing, among
others. In traditional slab gel electrophoresis, this
allows the use two separate separations on the same
gel, to improve the number of components that can be
resolved from one another (peak capacity). This is
20   done by separating components based on their
isoelectric point, using isoelectric focusing, then
rotating the gel 90 degrees, and separating the
components based on their size, using gel
electrophoresis. Other techniques for doing two-
25   dimensional separations have been devised, such as
described in US patents 5,496,460 and 5,131,998, and
international patents applications WO 02/40983 WO
00/57170.

30   The detection of biomolecules that have been
separated by capillary electrohoresis is an important
consideration. Typically, detection is performed
optically either by UV absorbance, or by laser induced

fluorescence (LIF) of a fluorophore that has been covalently bound to the analyte (called a 'tag' or 'label'), for the purpose of detection. It is often advantageous to add an internal standard or a control

5   to the sample, for simultaneous analysis on the same capillary. This allows one to control for subtle differences in injection and migration from capillary to capillary, and run to run. However, this requires the use of several labels of different wavelengths.

10 The use of a label may cause mobility shifts, which would prevent direct comparison of the sample to the standard or control unless this shift is matched for all of the labels used. In addition to shifting the mobility of analytes, labels may have different number

15 distributions among molecules of the same species. Using labels thus may lead to band broadening during the separation, which in turn may cause a loss in resolution. Further, because of the uncertainty in the number of labels to the number of analyte

20 molecules, labels reduce the ability to quantitate the analytes of interest.

There is a need for highly parallel, easy to use techniques such as multiplexed capillary gel

25 electrophoresis for the separation of proteins, peptides, or other biomolecules. There is also a need for advanced two dimensional separation systems for these molecules in a complex analyte. To increase sensitivity of detection for the highly parallel

30 electrophoresis separations, there is a need to detect endogenous (native) fluorescence of the analytes.

Summary of the Invention

Disclosed herein are methods and systems that can
be used, among other things, to separate and detect

5   various materials, in a parallel manner. The methods
and systems provide high resolution, high sensitivity
and high throughput detection of complex biological
samples.

10   In accordance with a first aspect of the
invention, there is provided a system and method to
perform separation and detection of components within
a sample. The system comprises an array of coplanar
parallel capillary electrophoresis tubes, each having

15   a first and a second end, said first ends being
arranged in a two-dimensional array having a spacing
corresponding to that of an array of wells of a
microtiter plate; an apparatus arranged to selectively
deliver sieving matrix and a selected one of a

20   plurality of liquids to said capillary tube second
ends; and a scanning means for exciting and detecting
radiation from said array of capillary tubes.

A preferred embodiment of the system utilizes a

25   size-based sieving matrix, such as LPA, dextran, or
galactomannans.

Another aspect of the current invention provides
a multiplexed capillary electrophoresis system and

30   method for the separation and detection of
biomolecules. The system comprises: an array of
coplanar parallel capillary electrophoresis tubes,
each having a first end and a second end, said first

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ends being arranged in a two-dimensional array having
a spacing corresponding to that of an array of wells
of a microtiter plate; an apparatus arranged to
selectively deliver sieving matrix and a selected one
5   of a plurality of liquids to said capillary tube
second end; and a scanning means for exciting and
detecting endogenous fluorescence radiation of the
biomolecules from said array of capillary tubes.

10   A preferred embodiment of the system utilizes a
size-based sieving matrix, such as LPA, dextran, or
galactomannans. A preferred scanning means includes a
laser capable of producing ultraviolet wavelength
light, such as a multiplied titanium sapphire laser
15   and harmonic generator.

Another aspect of the current invention provides
a method for separating and detecting components in a
complex biological sample by two-dimensional
20   separations, comprising: subjecting said sample to a
first separation and detection means; collecting
fractions into a fraction collection means while said
sample is being separated from said first separation
means; and subjecting more than one fraction
25   simultaneously to a second separation and detection
means, whereas the second separation and detection
means is based on a different property of the
component biomolecules being separated.

30   The method can further include the step of dye
labeling said complex biological sample before
subjecting said sample to the first separation and
detection means; or dye labeling said fractions of the

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complex biological sample after collecting said
fractions into said fraction collection means. The
method can also include the step of adding controls
labeled with mobility-matched dyes to the fractions
5   after said collecting step.

       The first separation and detection means consists
of HPLC, FPLC, ion exchange chromatography,
hydrophobic interaction chromatography, affinity
10   chromatography, isoelectric focusing,
isotachophoresis, capillary zone electrophoresis,
micellar electrokinetic chromatography,
electrochromatography, field flow fractionation, solid
phase extraction, liquid phase extraction, or any
15   other standard separation means. Preferably the
fraction collection means consists of a microtiter
plate. The second separation and detection means is a
highly parallel capillary gel electrophoresis system.
A preferred sieving matrix in the second separation
20   and detection means is galactomannans or dextran.

       Another aspect of the invention provides multi-
color detection for the simultaneous analysis of
controls and standards in the same channels as the
25   samples.

       The foregoing and other objects of the present
invention are explained in detail in the drawings
herein and the specification set forth herein below.
30

Brief Description of the Drawings

FIG. 1: Size-seiving based protein separation using multiplexed capillary electrophoresis with a galactomannan sieving matrix and LIF detection.

FIG. 2: Matching dye set separation of proteins.

FIG. 3A: Chromatogram of the HPLC dimension as first of two dimensional separation of rat liver proteins.

FIG. 3B: Zoomed view of area enclosed in the rectangle of the chromatogram shown in FIG. 3A.

FIG. 3C: Capillary electrophoresis separation of fraction enclosed in rectangle of the chromatogram shown in FIG. 3B.

FIG. 3D: Zoomed view of area enclosed in rectangle of the electropherogram shown in FIG. 3C.

FIG. 4: IEF-CE separation, the result from one fraction of the IEF dimension is shown as separated by CE.

FIG. 5: Two-color two-dimensional separation of *E. coli* protein extract separated by HPLC and CGE with a galactomannan sieving matrix.

FIG. 6A: Modified MegaBACE 1000™ instrument with titanium sapphire laser.

FIG. 6B: Modifications of MegaBACE 1000™ for the separation and detection of bioactive molecules using any method for excitation to produce endogenous fluorescence.

FIG. 6C: Detailed view of the detection region of the modified MegaBACE 1000™ system.

FIG. 7: Limit of Detection (LOD) plot for the endogenous fluorescence detector as shown in FIG. 6.

FIG. 8: Protein separation and endogenous fluorescence detection on a 96 capillary instrument as shown in FIG. 6.

5   Detailed Description of the Invention

The current invention is an instrument and system for the multiplex separation and detection of proteins, peptides, biomolecules and their conjugates,
10   small molecules and their conjugates, and polymers by electrophoresis and related techniques. The system has a plurality of capillaries or channels, of suitable material, such as glass or plastic. Electrophoretic separations are carried out in the capillaries or
15   channels, and detected using laser induced fluorescence (LIF) (either one or two photon processes). The LIF can be of fluorescently labeled molecules or the endogenous fluorescence of molecules.

20   Multiplex Separation with Galactomannans

One embodiment of the invention relates to multiplexed size-based electrophoretic separation of proteins and other biomolecules. Separation can be
25   achieved by either free zone electrophoresis, or electrophoresis with a sieving matrix, such as linear polyacrylamide (LPA).

In one embodiment, the separation and labeled
30   fluorescence detection of proteins on a 96 capillary instrument is achieved. A multi-capillary electrophoresis system, MegaBACE 1000™ (Amersham Biosciences, Sunnyvale, CA), is used in its unaltered

form. This instrument has a series of 6 arrays of 16
capillaries each, which couple to a high pressure cell
on one side of the array, resulting in the capacity to
fill each capillary with viscous matrices. After the
5 capillaries have been filled with fresh matrix, the
samples are loaded by electrokinetic means, the array
of samples is replaced with buffer, and a high voltage
is applied to provide the separation field.
Typically, this voltage is in the range of 8-20 kV,
10 although any voltage may be used. As the samples
progress down the capillaries, they pass a detection
region, in which laser-induced fluoresence detection
is accomplished. The LIF detection system of MegaBACE
1000™ has a confocal scanning fluorescence detector,
15 as described in US patent 5,274,240. This
fluorescence detector can collect up to four different
spectral channels of data per data acquisition cycle,
allowing for the simultaneous analysis of up to four
different chemistries per separation channel.
20

     US patent application number 09/946396, which is
incorporated herein by reference in its entirety,
discloses the process of purification for
galctomannans that is used in the instant invention.
25 The weight average molecular mass of the galctomannans
used is in the range of 10^5 and 3 $x10^6$. Galactomannans
having a molecular weight of at least 300,000 are the
preferred choice for sieving matrixes. The viscosity
and weight average molecular mass of galactomannans
30 can be reduced by the methods of ultrasonic treatment,
autoclaving, acid hydrolysis, and basic hydrolysis.
The preferred capillary column for protein analysis
has an interior cavity filled with a gel composed of

10 g/L galactomannans having a molecular mass of 7.7 x
10^5, 50 mM TRIS, 50 mM HEPES, and 4 mM SDS. Separation
is performed by introducing an aliquot of sample to
the capillary column, and applying an electric field
5   to the capillary column.

Using this method, 96 parallel size-based
separations of fluorescently labeled proteins are
routinely achieved. Typically, peak capacities are
10   about 50 for each capillary, and all of the components
of interest are adequately resolved. In cases where
the same separation was performed in all of the
capillarires, similar results are seen in all 96
channels. Thus, this method yields high-throughput,
15   reproducible separations of proteins and peptides.

Two Dimensional Separation Using CGE as the Second
Dimension

20   Separations of highly complex samples require
high peak capacity. For separations of biologically
active molecules the use of two dimensions of
separation is often necessary to resolve the large
number of components present in mixtures of either
25   biological or synthetic origin. The classic example of
this is the well-known art of 2D slab gel
electrophoresis, in which one dimension is isoelectric
focusing, and the other is size sieving. However, any
two separation techniques which have different
30   separation mechanisms may be coupled to provide a
better separation.

Another embodiment of the invention, referred to
hereafter as the 2D CGE device, provides an apparatus
and method for high resolution separation and high
sensitivity detection of proteins or other components
5   contained in biological samples, in a high throughput
manner.

There is provided a first dimension separation,
preferably performed by electrophoretic or
10 chromatographic means. Examples of separation
techniques that could precede size sieving include:
HPLC, FPLC, ion exchange chromatography, hydrophobic
interaction chromatography, isoelectric focusing,
electrochromatography, field flow fractionation, solid
15 phase extraction, liquid phase extraction and others.
This first dimension separation technique divides the
sample into a number of fractions where each fraction
may contain one or more components.

20 The invention further provides that each fraction
is collected into an interface device, such as a
microtiter plate. The interface device provides a
means for storage of the sample fractions, if desired.
An aliquot of each fraction can be used for the second
25 dimension separations, or other subsequent analysis.
If whole fractions will be used for the second
dimension separation, any needed modification to the
sample can be performed in the interface device. Such
modifications include any adjustment in solvents (if
30 desired), and labeling of the sample if it is not
labeled prior to the first dimension separation.
Alternatively, an aliquot of each fraction can be
transferred to a similar device, and used for the

second dimension analysis, while the remaining

aliquots can be used for other analysis, or archiving.

Additionally, controls and/or standards can be added

to each fraction. The controls and/or standards are

5   each labeled with a dye of a different fluorescent

emission wavelength than the dye used to label the

samples, but matched in mobility. This allows for a

direct comparison between the sample and the control,

and for the normalization of migration time for each

10   capillary. Such dyes are readily available, and

Figure 2 shows a separation of four proteins, each

labeled with three different dyes. The mobilities for

the dyes are very closely matched, so that the peaks

for each protein match well.

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     From the interface device, one or more of the

fractions (or an aliquot of the fraction, see above)

are simultaneously loaded onto the second-dimension

separation device, which further separates the

20   components within each fraction by capillary gel

electrophoresis (CGE). This second dimension

separation can be simultaneous for all, or for a

substantial number of fractions, of the sample being

analyzed. In the preferred embodiment, separation is

25   performed with a MegaBACE 1000™ system.

     The advantage of performing two dimensional

separations using the current system is that the time

frame for analysis of the second dimension need not be

30   extremely short compared to the time frame for

analysis of the first dimension, as with an integrated

2D device. This allows for greater flexibility in the

choice of each dimension, which otherwise would

constrain the first dimension to be very slow, to

allow for a reasonable separation time for the second

dimension, or the second dimension to be extremely

fast, which may not always be possible. It also has

5   the distinct advantage over techniques which collect

fractions from the first dimension separation, and

analyze them in serial, as the total analysis time is

greatly reduced, reducing the possibility of sample

degradation and increasing throughput.

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Multiplex Separation with UV-LIF Detection

The art of fluorescence detection for multi-

capillary electrophoresis systems for molecules which

15   fluoresce in the visible spectrum is well established,

as exemplified by US patent 5,274,240, 5,498,324 and

5,582,705. However, all of the multi-capillary

fluorescence detection systems available to date rely

on the use of fluorescent "tags", which require

20   derivatization of the molecules of interest. The

instant invention takes advantage of the endogenous

(native) fluorescence of certain molecules, which

allows for detection of the molecules without

derivitazation, and is a significant improvement in

25   the art of detection, as the derivatization can often

adversely affect the separation in a significant

manner. We refer to this detection system as the UV-

LIF system.

30   The UV-LIF system consists of a plurality of

capillaries, arranged in a coplanar manner, with a

confocal scanning fluorescence detector. This

invention is capable of detecting endogenous

fluorescence for multiple capillaries, using any of
several methods including UV and two photon
techniques. One such embodiment is described herein,
using a titanium sapphire laser and a armonic

5   generator capable of producing wavelengths in the
ultraviolet and specialty optics to deliver the laser
light in a tight spot, and to efficiently collect the
fluorescence emission. Alternatively, one could use
single wavelength lasers, such as frequency multiplied

10   gas lasers, frequency multiplied solid state lasers,
optically pumped solid state lasers, or multiple
wavelength alternative light sources such as mercury-
xenon lamps or diodes (should they become available).

15   FIG. 6 shows the instrument setup for the
invention, namely endogenous fluorescence detection of
bioactive molecules during separation. The
electrophoresis apparatus used was based on a MegaBACE
1000™ system, which was designed to do gel

20   electrophoresis of DNA. The electrophoresis component
of the system consists of arrays of capillaries which
are bundled and coupled into reagent tubes on the
anode end, and are distributed and coupled into a
microtiter plate on the cathode end. The detection

25   system in the MegaBACE 1000™ system is based on US
patent 5,274,240, and the current invention follows a
similar optical configuration, but is adapted to allow
for UV excitation, reflection, and fluorscent
emission. FIG. 6A shows a 96-capillary MegaBACE 1000™

30   system modified with a detection system of this
invention. In this embodiment, a titanium sapphire
laser (Spectra-Physics, Mountain View, CA) is used for
the excitation to replace the argon-ion laser

(Spectra-Physics) that is used in the commercial MegaBACE 1000™ detection system. The schematic for the laser induced fluorescence detector optics is shown in FIG. 6B. The implementation of the system for

5   transmitting UV and two photon sources of excitation energy involves the use of enhanced (protected) aluminum mirrors 10, 20, 30, and 40, UV sensitive diodes 50 for the detection of specular reflection during capillary positional registration, synthetic

10   fused silica and sapphire lenses 60, specially patterned reflective beam splitters 70, 80, and 90, and custom kinematic filter holders 100 for laser blocking at multiple wave lengths. These are described in the next four paragraphs.

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FIG. 6B presents the optical system for the invention. The solid line represents the incoming laser light, while the dotted line represents the fluorescent emission. The laser is a 1064 nm infrared

20   diode laser (Spectra-Physics,), which is doubled to 532 nm. This beam is then used to pump fluorescence processes in a titanium sapphire (Ti:Sapphire) laser, which can produce a wide range of wavelengths. In this embodiment, the Ti:sapphire is tuned to 840 nm

25   and tripled (using a tripling crystal) to 280 nm. This beam is then reflected off Mirror 1 (enhanced aluminum) (10) and directed to Mirror 2 (enhanced aluminum) (20) of the system. Mirror 2 is movable and allows the laser power to be monitored on the adjacent

30   power monitor before each run. Because the amplitude of the reflected laser light incident on photodiode so is greatest at the center of a capillary, a UV

enhanced diode is used to determine where the center
of each capillary lies.

Mirror 3 (30) is also an enhanced aluminum mirror
5   for optimal reflectance in the UV. The primary beam
splitter (80) before the scanning bench is a pattered,
UV enhanced aluminum mirror. It has a non-reflective
hole in the aluminum mirror to allow the beam to reach
the scan head. The returning beam is larger in
10   diameter and is passed by the reflective area of the
beam splitter to the laser blocking filters (100) and
eventually to the photo multiplier tubes (150, 160)
for detection.

15   After passing through the primary beam splitter
the beam travels to the low-mass scan head where it is
reflected (off Mirror 4, enhanced aluminum) to a
synthetic fused silica singlet lens (60). This beam
induces fluorescence in samples being separated in the
20   capillaries. Fluorescence from the samples in the
capillaries is collected by the same lens and
transmitted back to the primary beam splitter where it
is reflected into the detection area of the optical
bench.
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FIG. 6C shows the lightweight objective and
mirror mount and the scanning area. Reflectors and UV
enhanced lenses are necessary for the delivery of
laser light to the samples being separated. The
30   capillary window holder and capillaries are designated
in this figure.

The following examples are in no way exhaustive and merely represent some of the types separations possible utilizing the instrument and chemistries described.

Example 1: Protein separation by multiplex CGE

An unmodified MegaBACE 1000™ instrument was used for this separation. The 60 cm long capillaries were coated with linear polyacrylamide, then filled with a separation medium of 1% guaran sieving matrix, in 50 mM Tris, 50 mM HEPES, 4 mM SDS. Fluorescently labeled protein standards (Sigma, catalog number F3401) The labeled proteins were loaded onto the capillary columns by electrophoretic injection, with an injection time of 3 seconds at 10 kV. The protein standards were separated by electrophoresis over a period of 20 minutes at 12 kV. FIG. 1 shows a representative separation.

Example 2: Two-dimension separation of rat liver proteins by HPLC-CGE

In this separation, a MegaBACE 1000™ system was used to perform CGE as the second dimension separation, and an AKTA™ Explorer was used to perform HPLC as the first dimension. Protein samples were prepared from rat liver tissue which had been homogenized with polytrone in a buffer containing 8 M urea, 4% (w/v) CHAPS, 20 mM TRIS, 10 mg/mL dithiothreitol (DTT), and 17.4 mg/mL phenylmethylsulfonyl floride (PMSF). The samples were incubated for one hour, and then centrifuged to remove the insoluble material.

Two buffers were prepared for the separation:
buffer A: 10 mM phosphate buffer, and buffer B: 75%
acetonitrile in 10 mM phosphate buffer, pH 6.5. The

5   separation was performed on a Sephasil C4, 5 μm ST
4.6/100 mm column. The gradient used was as follows:
first, 4 ml 100% A were introduced, then a 34 ml
gradient to 100% B, and finally 12 ml 100% B. The
effluent was collected into 180 fractions of 200 μl

10   each in a microtiter plate well. These fractions were
then dried under reduced pressure, resuspended in 10
mM Tris, pH 8.5 buffer and labeled with the
succinimidyl ester of TMR for four hours in the dark.
The second dimension CGE separation was performed in

15   parallel on the MegaBACE 1000™ system. The fractions
were injected at 2 kV for 40 seconds and separated at
10 kV on 1% Guaran sieving matrix in 50 mM Tris, 50 mM
HEPES buffer and 0.1% SDS. FIG. 3A – 3D demonstrate
the two-dimensional separation of rat liver proteins.

20   The CGE separation of one fraction is shown in FIG. 3C
and 3D.

Example 3: Two-dimensional separation of rat liver
proteins by IEF-CGE
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Protein samples were prepared from rat liver
tissue as in the previous example. In this
separation, isoelectric focusing (IEF) was performed
on a drystrip (Amersham Biosciences, part number 17-

30   6002-44, 24 cm Immobiline Drystrip, pH 3-10), in the
conventional manner. The strip was then sectioned,
ground, and the proteins in each section was extracted
into 10 mM Tris 5mM SDS buffer (pH 8.5). The sections

were then analyzed in parallel by size sieving on a
MegaBACE 1000™ system (2 kV, 40 second injection, 10
kV run voltage), separated in 15% Dextran matrix with
a 10 mM Tris 5 mM SDS buffer (pH 8.5), on 60 cm long
5   capillaries. Shown in FIG. 4 is the CGE separation
profile generated from one IEF fraction.

           Example 4: Two-color two-dimensional separation
                  of E. coli proteins by HPLC-CGE
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         In this separation, a MegaBACE 1000™ was used to
perform CGE as the second dimension separation, and an
AKTA™ Explorer was used to perform HPLC as the first
dimension. Proteins were obtained by forming a pellet
15   from E. coli by centrifugation. The pellet was
resuspended in 8 M urea, 20 mM TRIS, 4% (w/v) CHAPS
with 0.1 mM PMSF. The cell suspension was sonicated
in an ice bath until clarified. 100 mg of DTT were
added to 10 mL of solution, and the solution was
20   incubated for 15 minutes, and then centrifuged.

         Two buffers were prepared for the separation:
buffer A: 10 mM phosphate buffer, and buffer B: 75%
acetonitrile in 10 mM phosphate buffer, pH 6.5. The
25   separation was performed on a Sephasil C4, 5 µm ST
4.6/100 mm column. The gradient used was as follows:
first, 4 ml 100% A were introduced, then a 34 ml
gradient to 100% B, and finally 12 ml 100% B.

30   The effluent was collected into 180 fractions of
200 µl each in a microtiter plate well. These
fractions were then dried under reduced pressure,
resuspended in 10 microliters of 10 mM Tris, pH 8.5

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buffer and labeled with the a 1 microliter of a
solution of 0.1 mg/mL of the succinimidyl ester of ROX
dissolved in DMSO for four hours in the dark. After
four hours, the volume was increased to 100

5   microliters with a 50 mM Tris-HEPES, 1% SDS buffer. A
set of molecular weight size standards were prepared
by labeling a solution 1 mg/mL in lactalbumin, trypsin
inhibitor, alcohol dehydroginase, and bovine serum
albumin with an excess of the succinimidyl ester of

10   rhodamine green dissolved in DMSO for four hours in
the dark. The standards were desalted on a Sephadex
G-20 column (Amersham Biosciences), diluted 100-fold,
and 3 microliters of the size standards were added to
each fraction of the sample.

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     The second dimension CGE separation was performed
in parallel on a MegaBACE 1000™ system. The samples
were injected at 2 kV for 40 seconds and separated at
10 kV on 1% Guaran sieving matrix in 50 mM Tris, 50 mM

20   HEPES buffer and 0.1% SDS.

     FIG. 5 demonstrates the two-dimensional
separation of proteins from the E. coli extract. The
trace at the bottom of the page represents the HPLC

25   separation, with UV assorption detection. Because UV
assorption detection is less sensitive than LIF
detection, not all of the proteins that are present
can be seen in this trace. The double trace on the
left-hand side of the figure represents the raw data

30   from separation of one of these fractions. Two of the
four spectral channels are shown in this trace (the
other two have been removed for clarity). The large
square block represents the full two-dimensional

separation. The bottom axis represents the HPLC
separation, with each fraction collected appearing as
a different vertical lane. Each parallel CGE
separation then proceeds from the bottom to the top of

5   the figure. Time is represented by the scan number.
Each scan represents about $1/100^{th}$ of a minute, so the
area shown represents from the 14th until the 30th
minute of the CGE separation, or about 16 minutes
worth of data. It is clear from this figure that there

10   is much more separation power using the two
dimensional separation method of the current
invention. It is also clear that data collected in
multiple spectral channels will allow for migration
time normalization of the sample (by the use of

15   standards) and the amount of each component (by the
use of controls).

                Example 5: A limit of detection plot
                  for the detection of tryptophan
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        To test the limit of detection (LOD) for the UV-
LIF system, we performed the following experiments.
Capillaries are mounted in a UV-LIF modified MegaBACE
1000™ system that accepts the endogenous fluorescence

25   detection, as shown in Fig. 6 and related descriptions
above. The capillaries were filled with dilute
solutions of tryptophan and were scanned at 280 nm
excitation. The signal minus the background divided
by the standard deviation of the background (S-B/SDB)

30   was calculated, and was compared between
concentrations in this plot. The limit of detection
is defined at the point where the signal to noise
ratio (S-B/SDB) reaches a value of three. In this
plot an LOD of $6x10^{-9}$ molar is demonstrated (Fig. 7).

Example 6: Separation of proteins using CGE with
endogenous fluorescence detection

A protein mixture containing 6 proteins was
5   analyzed on an UV-LIF system. To prepare this
mixture, 100 uL of a solution containing 5 g/L of each
protein was diluted with 10 uL 20% SDS, 10uL (100g/L)
DTT, 480 uL H_2O to a final volume of 600 ul. The final
concentration after dilution of each protein was:
10 insulin $1.7x10^{-6}$ M, α-lactalbumin $7.1x10^{-7}$ M, β-
lactoglobulin $5.6x10^{-7}$ M, cabonic anhydrase $3.4x10^{-7}$
M, ovalbumin $2.2x10^{-7}$ M, bovine serum albumin $1.5x10^{-7}$
M. This mixture was aliquoted to 10 uL per well in 16
wells of a 96 well microtiter plate (50 ug total
15 protein per well). This sample was injected at 10kV
for 10 seconds and run for 25 minutes at 10 kV with a
run buffer of 50 mM Tris, 50 mM HEPES and 0.1% SDS on
a UV-LIF modified MegaBACE 1000™ systemas shown in
Fig. 6 and described above. The signal to noise ratio
20 (signal minus background over the standard deviation
of the background) was 405 for β-lactalbumin. The
separation of the above proteins is shown in FIG. 8.
Bovine serum albumin was not observed due to
insufficient analysis time.

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The use of MegaBACE 1000™ and UV-LIF modified
MegaBACE 1000™ systems in the 1D and 2D separation of
proteins, peptides and other bioactive molecules not
only reduces the analysis time; it also offers
30 unparalleled peak capacity. It allows samples,
references/controls and standards to be run
simultaneously by using the matched dyes. These should
allow us to differentiate sample from dosed and un-

dosed histories, thus allowing for comparisons in drug development, toxicology, environmental effects and others.

5   It is apparent that many modifications and variations of the invention as hereinabove set forth may be made without departing from the spirit and scope thereof. The specific embodiments described are given by way of example only, and the invention is

10   limited only by the terms of the appended claims.